Chemical markers of human waste contamination: Analysis of urobilin and pharmaceuticals in source waters

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Abstract

Giving public water authorities another tool to monitor and measure levels of human waste contamination of waters simply and rapidly would enhance public protection. Most of the methods used today detect such contamination by quantifying microbes occurring in feces in high enough densities that they can be measured easily. However, most of these microbes, for example *E. coli*, do not serve as specific markers for any one host species and many can have origins other than feces. As an alternative, chemicals shed in feces and urine might be used to detect human waste contamination of environmental waters. One potential chemical marker of human waste is the compound urobilin. Urobilin is one of the final by-products of hemoglobin breakdown. Urobilin is excreted in both the urine and feces from many mammals, particularly humans. Source waters from 21 sites in New England, Nevada, and Michigan were extracted using hydrophilic-lipophilic balance (HLB) cartridges and then analyzed by high performance liquid chromatography-electrospray-mass spectrometry (HPLC-ES-MS). As a marker of human waste, urobilin was detected in many of the source waters at concentrations ranging from not detectable to 300 ng/L. Besides urobilin, azithromycin, an antibiotic widely prescribed for human-use only in the US, was also detected in many of these waters, with concentrations ranging from not detectable to 77 ng/L. This methodology, using both urobilin and azithromycin (or any other human-use pharmaceutical) could be used to give public water authorities a *definitive* method for tracing the sources of human waste contamination. The analysis and detection of urobilin in surface waters by HPLC-ES-MS has not been previously reported in the peer-reviewed literature.

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Introduction

        Public concern over cleanliness and safety of recreational and source waters has prompted researchers to look for rapid and accurate indicators of water quality. These waters can become contaminated with a variety of pollutants: industrial (*e.g.*, heavy metals, PCBs, dioxins, mercury), agricultural (*e.g.*, pesticides, antibiotics, nitrates, animal fecal matter), and anthropogenic (*e.g.*, human waste contamination, pharmaceuticals, personal care products). Some of these pollutants can cause a variety of illnesses in the various exposed organisms. Human waste (*i.e.*, feces, urine) released into water can carry a variety of diseases, (*e.g.*, polio, typhoid, and cholera). Sufficient pollution of an ecosystem can lead to environmental crises, such as, fish kills, red-tide blooms, and beach closings.

        Most of the methods used today detect such contamination by quantifying microbes occurring in feces in high enough densities that they can be measured easily. However, most of these microbes, for example *E. coli*, do not serve as specific markers for any one host species and many can have origins other than feces. Although, newer techniques for bacterial source tracking (BST) such as repetitive element polymerase chain reaction (re-PCR) procedures for DNA fingerprinting are being developed and applied, they too have limitations.[1] As an alternative, chemicals shed in human feces and urine might be used to detect human waste contamination of environmental waters. For example, some researchers have focused on developing methods for detecting and measuring fecal-derived sterols and bile acids as ways to measure human fecal contamination.[2-5] Others have reported using fecal sterols along with fluorescent whitening agents and linear alkyl benzenes as possible chemical indicators of human waste to differentiate between human and animal wastes in waters, even though these chemicals are not directly associated with feces.[6] One potential chemical marker of human waste contamination that has been reported is urobilin.[7] Urobilin is one of the final by-products of hemoglobin breakdown; it is excreted in both the urine and feces in many mammals, particularly humans.[8,9] The breakdown of hemoglobin to biliverdin is common to most animals, while the next step, the conversion of biliverdin to bilirubin, and subsequently to the urobilinogens (urobilin is one particular oxidized chemical form of urobilinogen) is unique to mammals.[10] One concern regarding the use of urobilin as a species-specific marker would be that other mammals produce urobilin. However, Collinder shows that compared with humans, cows, horses and pigs (three common farm animals) produce less than 10% of the urobilin produced by humans.[8] Miyabara *et al.* reported a positive correlation of urobilin with coliform levels, and a low correlation with chemical oxygen demand, making urobilin a good candidate as a chemical marker of water contamination by human waste.[11]

        Only two methods have been reported in the literature regarding the extraction and detection of urobilin as a chemical marker of fecal contamination.[12,13] These two methods use liquid chromatography with either UV-fluorescence detection[12] or photodiode array detection.[13] Both methods had limitations, where the samples had to be either manipulated before extraction via lyophilization and derivatization, or the extracts had to be hydrolyzed and derivatized before the urobilin could be detected by either UV or fluorescence. Another inherent limitation of the reported detection methodologies (UV and fluorescence) is their lack of specificity in the identification of either the analytes or other unknown compounds possibly present.

        To overcome these limitations, two methods of sample concentration, solid-phase extraction (SPE) C_{18} discs and SPE hydrophilic-lipophilic balance (HLB) cartridges, were investigated. The resultant extracts were analyzed by high performance liquid chromatography-electrospray-ion trap mass spectrometry (HPLC-ES-ITMS). Detection of urobilin by HPLC-ES-ITMS is very specific, and neither the sample nor the extract required hydrolysis, lyophilization, or derivatization, and the detection limits were comparable to the other two reported methods.

As a corollary to urobilin, finding a human-use pharmaceutical, along with urobilin, would confirm contamination from human waste as opposed to other sources, such as domesticated mammals, *e.g.*, dogs, cats, horses, cows. This paper explores the detection of one particular human-use pharmaceutical, azithromycin, in conjunction with urobilin, as pertinent chemical markers of human waste contamination. Azithromycin was chosen for multiple reasons: (1) it is approved only for human-use in the United States; (2) in the United States azithromycin prescriptions in 2004 ranked #8 overall; (3) previous research by the author had shown that this compound occurs in wastewater effluents[14]; and (4) azithromycin is amenable to the same analytical approach used for urobilin. The use of this new methodology could give public water authorities another tool to track sources and monitor and measure levels of human waste contamination, thereby improving public protection. The analysis and detection of urobilin in source waters by HPLC-ES-ITMS has not been previously reported in the peer-reviewed literature.

Experimental
Materials

d-Urobilin IX hydrochloride [(21H-biline-8,12-dipropanoic acid, 3,18-diethyl-1,4,5,15,16,19,22,24-octahydro-2,7,13,17-tetramethyl-1,19-dioxo-monohydrochloride, (4R, 16R)- (9Cl); CASRN 28925-89-5] was obtained from Frontier Scientific (Logan, UT, USA). The standard was a racemic mixture of four isomers, percentage of each is unknown. The urobilin purchased was prepared synthetically, a process by which four isomers are produced [structure (a) in figure 1 consists of two stereoisomers, with a designation of either Z- or E-] (figure 1). Azithromycin [(2R-(2R*,3S*,4R*,5R*,8R*,10R*,11R*,12S*,13S*,14R*)]-13-[(2,6-dideoxy-3-C-methyl-3-O-methyl-a-L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-ß-D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one; CASRN 83905-01-5] was obtained from U.S. Pharmacopeia (Rockville, MD, USA).

Stock standard solutions of urobilin and azithromycin were individually prepared in HPLC-grade methanol (Burdick & Jackson, Muskegon, MI, USA) and stored in darkness at $4^{o}C$.

The solvents used for both the extractions and the mobile phase for HPLC, were HPLC-grade methanol (Burdick & Jackson, Muskegon, MI, USA), glacial acetic acid (J.T. Baker, Phillipsburg, NJ), deionized water (NANOpure^{TM}, Barnstead, Dubuque, Iowa, USA), and ammonium acetate (Aldrich, St. Louis, MO, USA).

Sample collection

All water samples were collected using a "grab" sampling procedure, whereby a pre-cleaned 4-L amber glass bottle is submerged under water until filled. In theory these samples should then be returned to the laboratory for prompt extraction onto HLB cartridges (see next section for greater detail), as there is some concern about possible microbial degradation of the urobilin upon sitting *in situ* for more than 48 hrs.[7] During this study, the Duck Creek samples were collected and immediately returned to the laboratory for extraction and elution from the HLB cartridges; the Lake Michigan field samples were collected and shipped overnight, on ice, to the Las Vegas laboratory for extraction. Region 1 field samples (from Maine and Connecticut) were collected and sent to the U.S. EPA Region 1 laboratory for immediate sorption onto HLB cartridges.

Sampling sites

One set of samples came from a Southern Nevada creek, Duck Creek (Clark County),

that is not known to receive wastewater effluent. However, on either side of the creek, according to city planning maps, are housing developments that are dependent on septic tanks. This small creek had shown elevated nitrogen levels (personal communication with Dr. Jaci Batista, University of Nevada - Las Vegas), a possible indicator of nearby septic tank intrusion. A second set of samples was collected every other week between Memorial Day and Labor Day, 2004, from two recreational beaches on Lake Michigan that were near wastewater treatment plant (WWTP) effluent outfalls. A third set of samples was obtained from various rivers and streams receiving WWTP effluents throughout Maine and Connecticut.

Sample extractions
Solid phase extractions
SPE disks

Two different sorbents and geometrically different types of SPE were investigated.   The first type of SPE investigated were flat 48-mm diameter nu-phase^{TM} fiber C_{18} SPE disks from CPI International (Santa Rosa, CA) coupled with a CPI Accuprep 7000^{TM} manifold. The disks were prepared by rinsing with 10 mL each: methanol, methanol/1% acetic acid, and de-ionized (DI) water. Water (2-L) was adjusted to approximately pH 3 with HCl (12N), then allowed to flow by gravity through the prepared SPE disks for 40 minutes. After extraction, the disc(s) (it is recommended to not fully dry the discs) are eluted with three 10-mL volumes of 99% methanol/1% acetic acid. The eluants are blown down to 0.5 mL using a TurboVap^{TM} (Zymark Corporation, Hopkinton, MA, USA), water bath set at 25 ^{o} C, and N_2 flow at 4 psi initially. As the N_2 flow is gradually increased to 13 psi, and as the 20 mL of eluant is slowly concentrated, it is recommended to rinse the walls of the extraction tubes at least 2 or 3 times (using 99% methanol/1% acetic acid) during the blow-down procedure. The final eluant is analyzed by HPLC-ES-ITMS.

SPE HLB cartridges

The second type of SPE explored were OASIS HLB cartridges, 6-mL capacity, 0.2 g, 30-µm, obtained from Waters Corporation (Milford, MA), using a Supelco (Bellefonte, PA) 12-port SPE vacuum manifold for processing the cartridges. The cartridges were prepared by rinsing with 5 mL methanol, followed by 2 x 5-mL rinses of DI water. The cartridge was then loaded with 500 mL of water sample, and the water sample adjusted to < 3 pH, with 12N HCl, before processing. A constant flow of 3 to 4 mL/min is maintained throughout the loading process, using a constant pull by the vacuum pump. When finished loading, the pump is turned off; it is not necessary to dry the cartridges. Collection tubes are placed under the cartridges, and the analytes are eluted using 4 x 5 mL of methanol/1% acetic acid. The eluants are blown down to 0.5 mL using a TurboVap^{TM} (Zymark Corporation, Hopkinton, MA, USA), the water bath set at 25° C, and the N_2 flow set at 4 psi initially. The N_2 flow is gradually increased to 13 psi as the 20 mL of eluant is slowly concentrated, it is recommended to rinse the walls of the extraction tubes at least 2 or 3 times (using 99% methanol/1% acetic acid) during the blow-down procedure. The final eluant is analyzed by HPLC-ES-ITMS.

Region 1 extracts

The Region 1 HLB cartridges were initially elueted with two successive 3-mL rinses of 10% methanol/90% MTBE for a separate analysis of estrogenic and endocrine disrupting compounds (bisphenol A, nonylphenol, ethynyl estradiol, estradiol, and estrone) by Region 1. The extracted cartridges were then wrapped in foil and stored < 0° C, until subsequent extractions by this experiment (elution using 99% methanol with 1% acetic acid) could proceed nearly 2

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years later. Prior to extracting the cartridges with the 99% methanol:1% acetic acid and sending the extracts to our laboratory for HPLC-ES-ITMS analysis, Region 1 performed an HLB azithromycin recovery experiment using this dual elution scheme. The recovery results were 76% recovery of the azithromycin (16% relative standard deviation), and 8% of the azithromycin is lost to the MTBE fraction. At this time, no holding time criteria have been established for either urobilin or azithromycin on the HLB cartridges while frozen, therefore the final concentrations could be either higher or lower.

HPLC-ES/ITMS analysis
Liquid chromatography

The separations were performed using a Restek Allure C_{18}, 5-µm particle size, 150 x 3.2-mm liquid chromatography column (Bellefonte, PA), with a flow rate of 0.40 mL/min, and a 40:60 split after the column, such that 40% of the flow (160 µL/min) goes to the ES-ITMS. For example, if the injection volume on-column was 20 µL, then the volume entering the ES-ITMS is only 8 µL, due to the 40:60 split. The gradient elution conditions were: 100% mobile phase A (hold for 1 min) to 100% mobile phase B (hold for 5 min) over a 20-min gradient, with a 5 min equilibrium between runs. Mobile phase A: 99% water/1mM ammonium acetate/0.1% acetic acid/1% methanol; mobile phase B: 98% methanol/1mM ammonium acetate/0.1% acetic acid/2% water.

Electrospray-ion trap mass spectrometry

A ThermoQuest Finnigan LCQ^{TM} (San Jose, CA), configured with an ES ion source, was used to detect the pharmaceuticals. The LCQ uses an ITMS detector that performs real-time mass analyses of LC eluents over a mass-to-charge ratio range of 50 to 2000. The LCQ was run in the positive ionization mode, the voltage applied to the ES needle ranged from 4.2 to 4.6 kV (dependent upon the optimized response of the ions of interest), the heated capillary was set at 215°C, and the sheath gas was set dependent upon the optimized response of the ions of interest [these sheath gas values could range from 30 to 60, where the range is arbitrarily set by the manufacturer from 25 to 100 (no units)]. When using the full-scan mode for screening the extracts, the ITMS was scanned from 120 to 830 amu (full-scan mode) in 3 µscans with an ion injection of 200 ms. Two other modes, selected ion monitoring (SIM) and collision-induced dissociation (CID), were used for quantifying and confirming the analytes.

MS/MS experiments - SIM and CID
Selected ion monitoring

SIM is a mass spectrometric procedure where only those ions of interest are monitored (*e.g.*, urobilin mass 591 m/z, azithromycin mass 749 m/z), and the rest are filtered out. In all cases, urobilin and the other pharmaceuticals detected by SIM were confirmed by MS/MS. The MS/MS confirmation is necessary due to the lack of specificity created by using only one ion for detection in the SIM mode.

Collision induced dissociation

The LCQ can be used to perform CID experiments (MS/MS or MS[n]) in the ion trap. The precursor ion of interest is isolated in the ion trap (using SIM), and voltages are applied to the trapped ions inducing collisions and subsequent product ions (ions that are produced from the precursor ion). The collision energy (CE) is related to the precursor ion and the amplitude of the resonance excitation radio frequency (RF) voltage. CE is the percent of a maximum voltage used to accelerate ions into collisions. Each ion trap has a unique slope and intercept of the

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amplitude of the RF, but each manufacturer ensures that these values are normalized to a percent of the amplitude voltage, thereby guaranteeing (all other conditions the same) that MS^n spectra are reproducible from instrument to instrument. For the ion trap used in this research the slope is 0.001126 V/µ and intercept is 0.4 V. The collision energies (CE) depend on the precursor ion selected, usually the most abundant ion from the full-scan mode spectra, and the amount of fragmentation desired for confirmation; in the case of urobilin and azithromycin, a CE of 25% was determined to give the optimal amount of information, product ions plus remaining precursor ion.

Calibration, blanks, and HPLC-ES-ITMS quantitation

For each set of HPLC-ES-ITMS analyses, a calibration curve consisting of duplicate or triplicate standard solutions was produced. Two standards were analyzed at the beginning of each day of operation; then a series of solvent blanks [until no carryover was detected, or the signal was well below the limits-of-detection (LOD)], then samples (field blanks and samples) and a final standard were analyzed in that order. An external standard calibration procedure was used, the mechanics of this procedure are outlined in EPA's Solid Waste-846 manual, 8000B, section 7.4.2.1 [available at http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8000b.pdf]. Either an individual ion using SIM or the product ion from CID was used for quantitation purposes. For SIM, the areas under the SIM ion chromatogram peaks are quantified using a manual quantitation procedure, as allowed by the software on the LCQ. When using CID, a CE of 25% for urobilin and azithromycin was selected to standardize the ratio of product ion (urobilin, 343 m/z; azithromycin, 591 m/z) to precursor ion (urobilin, 591 m/z; azithromycin, 749 m/z), both for standards and samples. The product ion was monitored and the area under the product ion was quantified, as explained in the SIM procedure. In analyzing extracts by both procedures, no significant difference was seen in quantitation values calculated by either SIM or CID.

Limits-of-detection

The ES-ITMS LOD were determined for urobilin and azithromycin. LOD is defined as the lowest concentration of an analyte that an analytical process can detect and is located at 3s (s = standard deviation) above the gross blank signal. Using regression analysis on the data obtained from analyzing urobilin at four different concentrations, using full scan mode, the LOD for urobilin was calculated as 32 pg (r^2 = 0.999) on-column, and 340 pg for azithromycin.[15]

Results and discussion

Extraction comparisons - SPE C_{18} discs versus HLB cartridges

At the beginning of this research project, SPE C_{18} discs were investigated for their extraction efficiency. The percent recoveries obtained from the discs were around 50% (table 1). Other research had suggested that a different sorbent material might be more appropriate and better recoveries obtainable.[16] During the comparison process it was noted that the HLB cartridges were not sensitive (spiked recoveries remained consistent) to inadvertent drying during the extraction process, unlike the SPE C_{18} discs, which are very sensitive to drying. The HLB cartridges proved easier to use, were more rugged (less worry about drying during extraction), required less sample (500mL vs 2-L) for comparable recoveries, used less extraction solvent (20mL vs 60mL), and recoveries were generally better (> 75%). Therefore, the HLB cartridges were the final choice for use for extraction of urobilin from source waters.

The efficiency of the urobilin extraction methodology was determined by spiking both DI waters and natural waters with 1 µg/L of urobilin, and obtaining 70% efficiency or greater (using

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the HLB sorbent) recovery (table 1). In actual samples, urobilin was routinely and accurately detected in the low-ppt range, 10 to 300 ng/L (table 2) using the 3s to 10s range as region of detection and >10s as the range of quantitation, above the gross blank signal as a guide.[15] In comparison, the HPLC-fluorimetric detection method described by Miyabara *et al.*[12] reports a detection limit of 0.2 µg/L, and the HPLC-diode array detection method described by Piocos *et al.*[13] reports a detection limit of 100 µg/L.

The precision of the laboratory generated duplicates (table 2: samples 26900a and 26900b, and 26902a and 26902b) indicate that the quality control of the analytical methodology (extraction and detection) is acceptable.

HPLC-ES-ITMS spectra results

HPLC-ES-ITMS analysis of the urobilin IX hydrochloride standard (MW = 626 daltons) produced one distinct chromatographic peak, retention time of 21 min. The primary ion detected for urobilin is mass 591 m/z (100% intensity), the $(M + H - HCl)^+$ ion. Present at the same retention time as mass 591, but also present at lower intensities are several other masses: mass 592 (34% intensity); mass 343 amu (7% intensity); and a cluster of masses at 466-467-468 amu (4% intensity). Mass 592 is the contribution from the C_{13} isotopes from urobilin's 33 carbons. Mass 343 is attributable to the loss of two of the nitrogen rings from the parent ion, $[M - 2(C_7H_{10}NO) + H - HCl]^+$. Mass 466 is attributable to the loss of one nitrogen ring, $[M - C_7H_{10}NO - HCl]^+$, and mass 467 and 468 are attributable to $[M - C_7H_{10}NO + H - HCl]^+$ and $[M - C_7H_{10}NO + 2H - HCl]^+$, respectively. The ITMS was used in the CID mode to induce fragmentation in mass 591, such that a "fingerprint" spectrum could be made and aid in identification of urobilin in complex wastewater and natural water matrices. The results of the CID studies gave exactly the same ions produced without CID, only the product ions produced were of a higher intensity (15 to 20% of the precursor ions) (Figure 2). Two other analytes were confirmed as present in the samples, azithromycin and methamphetamine. Their ES and CID spectra have previously been reported in Jones-Lepp *et al.*.[14]

Application of method to real-world samples

There is concern regarding raw sewage intrusion into recreational beach areas, well waters, and drinking water sources. One of the main reasons that urobilin was chosen is that it is a natural by-product of human waste (feces and urine), and therefore a potential marker of intrusion of human sewage into natural waters. Several sites were investigated in the Southwest, Great Lakes, and New England, that could possibly have sewage contamination problems. A known human-use pharmaceutical, azithromycin (antibiotic) was also screened for, with the reasoning that if both urobilin and azithromycin could be detected in the source waters then the contamination could more definitively be identified as human in origin.

Characterization of HPLC-ES-ITMS analyses

Southern Nevada. The samples collected from Duck Creek, Nevada, were returned without delay to our Las Vegas laboratory for immediate extraction. These samples showed levels of urobilin at 18 ng/L. The finding and levels of urobilin in Duck Creek were consistent with the findings of Miyabara *et al.* of contaminated streams and rivers in Japan[11], and were also logical with regards to the septic tanks that surrounded the sample collection area. While azithromycin was not detected in these samples, methamphetamine (an illicit drug) was detected, and confirmed by MS-MS. While finding methamphetamine was unexpected, it had been detected previously in earlier analyses of waters from this site, using the SPE C_{18} disc methodology (the water samples for the C_{18} discs method were pH adjusted to a more basic condition). Previous

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work had reported methamphetamine in this metropolitan area's waste water sewage effluent.[14]

Great Lakes. Samples were collected every other weekend (on Sunday), from Memorial Day to Labor Day, 2004, from the two Lake Michigan sites (Silver Beach and Washington Park Beach), kept cold (via ice packs), and shipped on Monday (overnight delivery) to our Las Vegas laboratory for subsequent extraction and analysis. There was no urobilin detected in any of these samples.

New England. The last set of samples, from New England (Maine and Connecticut), were collected and sorbed onto HLB cartridges by Region 1 scientists during the fall of 2002 and the summer of 2003, the cartridges were kept frozen, $< 0^{\circ}$C, until extraction in September 2004 (see experimental section for greater detail). The subsequent Region 1 extracts were sent to our laboratory analysis. The samples analyzed from Region 1 showed varying amounts of urobilin and azithromycin.

Sample AA26903, Royal River Yarmouth Landing, Maine, had the highest amount of urobilin present at 316 ng/L, and a trace of azithromycin, < 1 ng/L, both positively identified via MS-MS. The high levels of urobilin in this sample may be due to the sample site being located in a tidal area approximately 0.6 km across the bay, and slightly north, from the Yarmouth WWTP, as well as having a large moored boating area. Just prior to the sampling time (November 2002), the City of Yarmouth had issued a bacterial advisory for drinking water samples from the local aquifer (August 2002 and October 2002).[17] Their analyses had shown higher than normal coliform bacteria in the drinking water supply. Sample AA26902, Yarmouth WWTP (secondary treatment, avg. flow 1.3 millions of gallons per day, mgd) effluent outfall, did not show the high levels of urobilin as the Yarmouth Landing sample. Methamphetamine was detected in both the sample and sample duplicate (avg 5 ng/L) of the Yarmouth effluent; again this is consistent with previous finding of methamphetamine in another US city's WWTP effluent, though the other city had a much larger population base and higher effluent flow rate (tertiary treatment, avg. flow 50 mgd) than the Yarmouth WWTP.[14] Because the Yarmouth bay area had a larger level of urobilin than that from the WWTP outfall, it can be inferred that additional sources of human waste might be entering into the Yarmouth Landing bay area. It is possible that the other sources are from marina toilets, leaking septic tanks, and possibly straight-piped raw sewage. A similar-type of sample (AA26909, Hampden boat landing) was collected from a different harbor area, which also has a large boat mooring area, and it too showed elevated levels of urobilin, 52 ng/L. The nearest WWTP, the Bangor WWTP, is approximately 2 miles away, again leading to the possibility that the source of the high levels of urobilin are from sources other than the Bangor WWTP.

Sample AA26900 had the highest levels of azithromycin detected in the New England samples, 77 ng/L, consistent with previous findings in a similar type of WWTP (tertiary) in the Southwest.[14] Tertiary treatment does not seem to efficiently remove azithromycin, as evidenced by its detection at both the Sanford and Portland WWTPs, as they both have similar environmental loadings, 0.5 kg/yr and 0.6 kg/yr, respectively. Sample AA26907 was collected less than 0.3 km away from the S. Portland WWTP, in a tidal flat area. Both urobilin and azithromycin were detected in this sample, with levels of 16 and 1.5 ng/L, respectively, demonstrating the probability that these compounds can migrate away from their point of origin. This effect was also seen between the Sanford WWTP site and the Mousam River site (AA26901). The level of azithromycin was 77 ng/L at Sanford, but drops off to 47 ng/L at the Mousam River site, which is 400 ft downstream from the Sanford WWTP.

None of the Connecticut samples were collected directly from WWTPs. Most were

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located 1 mi or greater from WWTP sewage outfalls, yet both urobilin and azithromycin were detected in most of those samples. The highest amount of azithromycin detected in the Connecticut samples was 39 ng/L, from sample AA29826. This sample was collected 1.5 mi downstream from the Bristol WWTP (secondary treatment, avg. flow 10 mgd), and no urobilin was detected. The second highest amount of azithromycin detected (34 ng/L) was from sample AA29827, which was collected 3.25 mi from the nearest WWTP, Danbury WWTP (secondary treatment, avg. flow 15.5 mgd); urobilin was also detected, but at a very low level near the LOD. The Danbury WWTP effluent empties into a swamp before it enters into the Still River, the makeup of the Still River is 90% treated sewage.

A very small amount, 2 ng/L, of azithromycin was detected in sample AA29828, which was collected 0.5 mi downstream from a senior housing condominium complex, which has its own small WWTP. The makeup of the Pomperaug river at the collection point is approximately 30% sewage effluent.

Sample AA29831, collected 2.5 miles downstream from the Naugatuck WWTP on the Naugatuck river, had 17 ng/L of urobilin present. The Naugatuck river, at this sampling point, contains approximately 80% sewage effluent; it receives effluent form failed raw sewage collection systems. The last sample in the New England data set, AA29832, showed 22 ng/L of urobilin present, and was collected 2.5 miles downstream from the Meriden WWTP on the Quinnipiac river, which at this sampling point is made up of approximately 40% sewage effluent.

Overall, the amount of urobilin at the various New England sites ranged from not detected (nd) to 316 ng/L, and azithromycin from nd to 77 ng/L. These azithromycin results are consistent with findings in US wastewater effluents from a previous study using time-weighted samplers.[14]

The environmental significance of finding azithromycin in most of the waters sampled, at low-level concentrations, is uncertain at this time. Recently, it has been reported in the literature the occurrence of antibiotic-resistant bacteria in waters receiving wastewater effluents.[18,19] This resistance probably originates from gene-transfer from the shedding of bacteria that have been exposed to therapeutic concentrations, but the consequences of organisms exposed to antibiotic concentrations orders of magnitude lower cannot be discounted.[20]

The results from this study would indicate that urobilin could possibly be used as an indicator of the presence of human waste, and that the presence of a human-use pharmaceutical gives greater credence to that result. The methodology used in this study proved itself to be sensitive (LODs in the ng/L range), quantifiable (82% recovery, 26% rsd), and specific (with the use of MS/MS). The state of development of this methodology is such that it could be used by public water authorities as another tool for tracking the source of human waste contaminants into source waters. With further refinements, such as looking at correlations between urobilin levels and coliform levels, this methodology may be able to indicate impairment of recreational and source waters from human waste. The author intends future analyses of source waters for urobilin, nitrate and coliform levels, and evaluation of principal component analysis (PCA) to determine correlations among the data. Doing so will strengthen the use of this analytical tool for public protection.

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Table 1. Comparison of SPE C_{18} vs HLB spiked recoveries

of urobilin in deionized and source waters

DI waters (1 µg/L) recoveries %		Natural waters (1 µg/L) recoveries %	
C_{18}[a]	HLB[b]	C_{18}	HLB[c]
49 (35% rsd)	77 (22% rsd)	no data	82 (26%rsd)

[a] n= 8; [b] n= 4; [c] n= 4

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Table 2. Concentrations of urobilin from source waters

Sample	Urobilin ng/L	Azithromycin ng/L	n
Duck Creek, Las Vegas, NV[1]	18	nd	2
Lake Michigan beach site 1 (Silver Beach)			
June 29, 2004	nd	nd	3
July 13, 2004	nd	nd	2
July 27, 2004	nd	nd	2
August 17, 2004	nd	nd	2
September 8, 2004	no sample		0
Lake Michigan beach site 2 (Washington Beach)			
June 29, 2004	no sample		0
July 13, 2004	nd	nd	2
July 27, 2004	nd	nd	2
August 17, 2004	nd	nd	2
September 8, 2004	nd	nd	2
Region 1 (Maine, Connecticut)			
AA26900a	†	77	1
AA26900b laboratory duplicate	†	75	1
AA26901	33	47	1
AA26902a [2]	11	nd	1
AA26902b laboratory duplicate [2]	15	nd	1
AA26903	316	†	1
AA26904	11	nd	1
AA26905	21	†	1
AA26906	295	41	1
AA26907	16	†	1
AA26909	52	4	1
AA29823	†	13	1
AA29824	nd	nd	1
AA29825	16	5	1
AA29826	nd	39	1
AA29827	†	34	1
AA29828	nd	2	1
AA29829	nd	15	1
AA29830 field duplicate of 29829	42	23	1
AA29831	17	nd	1
AA29832	22	nd	1
Control blank	nd	nd	1

nd = non-detect; † positive MS/MS identification, but below LOQ;
[1] Methamphetamine detected: 7 ng/L.
[2] Methamphetamine detected: 3 ng/L and 7 ng/L.

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Figures

(1) Isomeric structure(s) of urobilin IX hydrochloride.

(2) Product ion chromatogram and mass spectrum of urobilin IX hydrochloride.

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Figure 1 Urobilin IX hydrochloride [21]
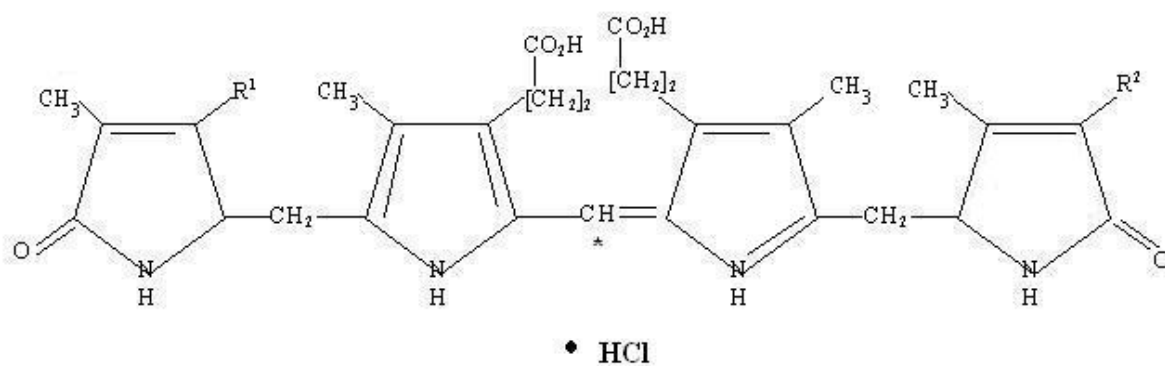
(a) $R^1 = R^2 =$ Ethyl (* Z- or E- isomer)
(b) $R^1 =$ Vinylic, $R^2 =$ Ethyl
(c) $R^1 =$ Ethyl, $R^2 =$ Vinylic

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Figure 2. Product ion chromatogram and mass spectrum of urobilin IX hydrochloride

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